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Morphological Properties of Mesenchymal Stem Cells Derived from Bone Marrow of Rhesus Monkeys

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Abstract: To establish an *in vitro* system for isolating and culturing the mesenchymal stem cells (MSC) of Rhesus monkeys, and to provide research data for its further application, the bone marrow of Rhesus monkeys was collected and separated by gradient centrifugation to discard most of the blood cells. The MSC contained in the monocyte centrifuging layer was obtained and cultured in Dulbecco's modified media (low glucose, L-DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1 ng/ml basic fibroblast growth factor (bFGF). The non-MSC was screened out by continuously renewing the medium. A passage culture was undertaken while the MSC monolayer formed. The spindle-shaped MSC formed a monolayer after 18 days of primary culturing, and the cells appeared in an oriented array with a swirling and irradiating growth trend. In the anaphase of passage culture, the cell proliferation rate was decreased and the morphology changed into triangular, polygon and flat appearance. These results suggested that mesenchymal stem cells (MSC) of the Rhesus monkey can be passaged *in vitro* with the established optimized culture system.

Key words: Rhesus; Mesenchymal stem cell (MSC); Cell culture

恒河猴骨髓间质干细胞的体外分离培养及形态学特征分析

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摘要: 探索恒河猴骨髓间质干细胞 (MSC) 的体外分离培养方法, 为其应用提供实验基础。取恒河猴骨髓细胞悬液, 经梯度离心去除大部分血细胞, 取含有 MSC 的中间单核细胞层, 在含 10%胎牛血清及 1 ng/mL 碱性成纤维细胞生长因子的 L-DMEM 中培养扩增, 并不断换液去除杂细胞, 经过 18 d 的原代培养, 获得呈致密单层生长的 MSC, 其形态为较规则的长梭形细胞, 排列有方向性, 呈现一定的漩涡状、辐射状生长趋势。将原代细胞以 1:2 传代, 传代培养后期, 细胞增殖速度逐渐变缓, 细胞形态逐渐出现三角形、多边形及扁平宽大形等不规则形态。结果显示, 恒河猴骨髓间质干细胞可在体外进行传代培养, 但需进一步优化其培养条件。

关键词: 恒河猴; 骨髓间质干细胞; 细胞培养
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Bone marrow is the major source of stromal cells and adult hematopoietic stem cells (HSCs) that renew circulating blood elements; these cells can also be found in other tissues. Adult bone marrow also contains mesenchymal stem cells (MSCs). MSCs are thought to be multipotent cells that can replicate as undifferentiated cells, and have the potential to differentiate to lineages of mesenchymal tissue, including: bone, cartilage, fat, tendon, muscle, nerves and marrow stroma (Pittenger et al, 1999; Minguell et al, 2001). Thus,

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the apparent multipotent nature of MSCs makes them excellent candidates for tissue engineering. *In vitro* and animal implant studies have indicated that animals either possess multipotent MSCs or the populations studied are mixtures of committed progenitor cells, each with a restricted potential (Pittenger et al, 1999). We report here the isolation, expansion and characterization of the multipotent Rhesus monkey MSC (rhMSC).

1 Materials and Methods

1.1 Materials

All tissue culture plastic ware was from Fisher. Fetal bovine serum (FBS) was purchased from Hyclone (Life Technologies, USA). Dulbecco's modified media (low glucose, L-DMEM), trypsin/EDTA and antibiotic were purchased from Gibco (Gibco, USA). Ficoll were purchased from Pharmacia.

1.2 Isolation of MSCs from Rhesus monkey bone marrow

Adult breeding Rhesus monkeys (*Macaca malatta*) were obtained from the Primate Research Centre, Institute of Medical Biology. Healthy monkeys aged three years old were used for this study, conducted in accordance with Chinese guidelines for animal welfare. MSC was isolated from 2 ml aspirates from the iliac crest of the monkeys, which were anesthetized with isofluorane. The aspirates were washed with 15 ml of PBS and layered over 10 ml of ficoll. After centrifugation at 2 500 × *g* for 25 min, the mononuclear cell layer was removed from the interface and resuspended in L-DMEM. The cells were washed twice using medium at 1 500 × *g* for 25 min at 4℃. The cell pellets were resuspended in L-DMEM and the cell concentration determined using counting slides. MSCs were plated at a concentration of 1×10^6/ml in a 6-well culture plate.

1.3 Culture and expansion

Following plating, MSCs were maintained in a humidified incubator at 37℃/5% CO_2 in L-DMEM, supplemented with 10% FBS and 1 ng/ml bFGF. The medium was changed after 24 h to remove nonadherent cells and twice weekly thereafter. Cells were passaged with 0.25% trypsin/0.1% EDTA upon reaching 90% confluency and expanded until passage 5, whereupon they were analyzed.

2 Results

2.1 Rhesus MSC primary culture

The bone marrow isolates suspended in culture were removed by changing the medium at 24 h. In the first five days, the adherent cells grew in a round shape with few morphological changes. Under an inverted-phase contrast microscope, morphological conservation was observed. Renewing the medium every third or fourth day thereafter, the results showed that the parental MCS was characterized by an enlarging appearance, inferring that it supplemented its growing ability by adjusting to the *ex vivo* culture. At day 9, a spindle shape was observed in well-spread colony-forming cells (Fig. 1 A). Subsequently, most adherent cells grew in a spindle shape characteristic of fibroblasts at day 12 (Fig. 1 B). By day 15, the MCS was duplicating rapidly, and the cell morphology was mainly spindle-shaped with a triangular appearance (Fig. 1 C). Observations on day 18 revealed that the cell monolayer was forming with constant orientation and a whirling tendency (Fig. 1 D), which means the cells have the potency to be passaged in ratio of 1:2.

2.2 Rhesus MSC passage culture

Passage 2 of the mesenchymal cells was characterized by the ability to proliferate in culture with an attached well-spread morphology, reaching confluence on day 6 (Fig. 2 A). Basically, passages 2, 3, 4 and 5 have a relative rapid adherence occuring within 24 h. However the cell proliferation rate decreased in passages 4 and 5 compared with the earlier passages. Microscope observations showed passage 3 cells remain fast growing, with cell-clones forming by day 2 and reach 75% confluence by day 5. The cell morphology displayed non-orientation and a whirling tendency. In passage 4, cells continued to duplicate rapidly, however were polygon-shaped (Fig. 2 B). After splitting on day 6 (passage 5), the cell proliferation obviously slowed down. In this case, few typical spindle-shaped cells can be observed under a microscope, and more triangular, polygon or flat cells were found in the field.

3 Discussion

Mesenchymal stem cells are thought to be multipotent cells, which are present in adult marrow. They can replicate as undifferentiated cells and have the potential to be induced to differentiate exclusively into adipocytic, chondrocytic or osteocytic lineages (Prockop et al, 1997). It has been reported that MSCs can be isolated by their adherence properties to the plastic surfaces of culture flasks, displaying fibroblast-like growth characteristics and increased proliferation (Friedenstein et al, 1976).

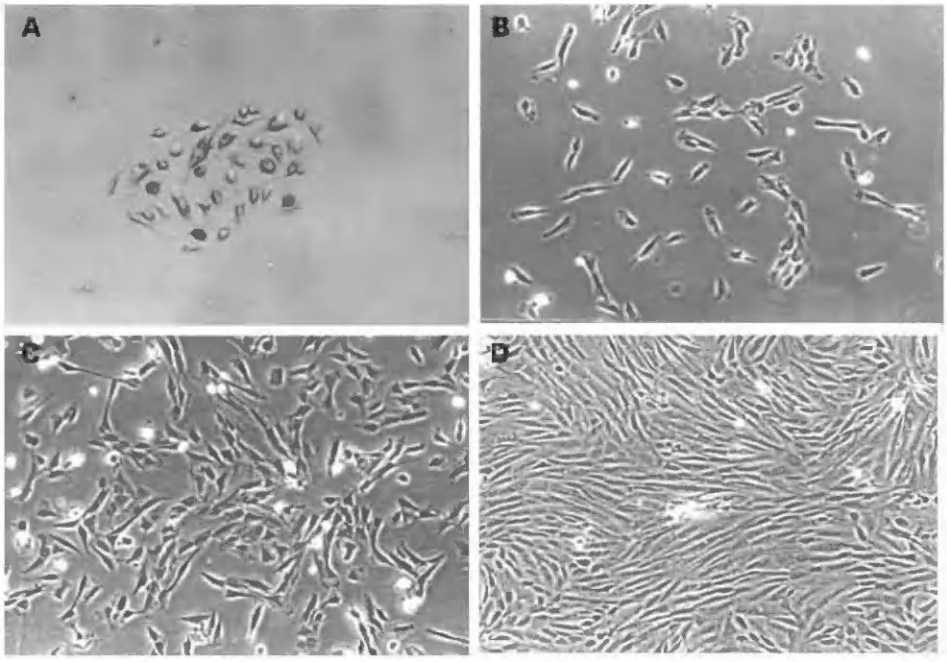

Fig. 1　Morphology of Rhesus MSC primary culture (× 100)
A. 9 days; B. 12 days; C. 15 days; D. 18 days.

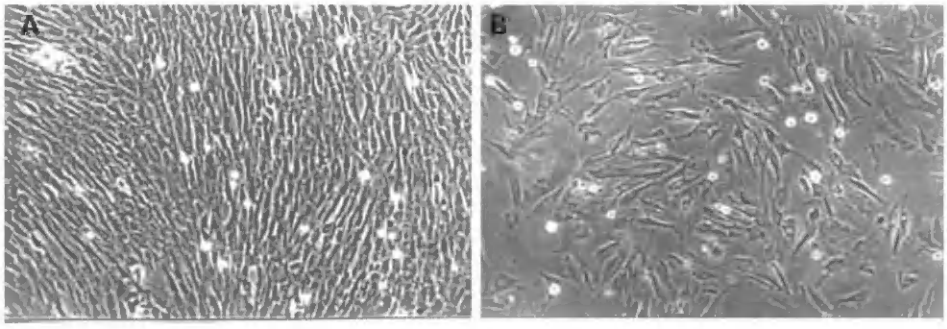

Fig. 2　Morphology of Rhesus MSC passage culture (× 100)
A. Passage 2; B. Passage 4.

Bone marrow traditionally has been viewed as the home of hematopoietic stem cells. It is also known to contain MSCs as part of the stromal fraction. MSCs represent a small, non-hematopoietic subpopulation of cells that reside in the bone marrow, and have the potential to serve in tissue-engineering applications involving fat, bone, cartilage, and possibly nervous tissue. The multipotentiality of bone marrow-derived MSCs makes them promising candidates for mesodermal defect repair and disease management (Conget & Minguell, 1999; Majumdar et al, 1998; Pereira et al, 1995; Dennis et al, 1999). Due to their ability to secrete cytokine, MSCs can act as breeding cells in supporting and regulating hematopoietic and embryo stem cells for bone morrow transplant and gene therapy (Minguell, 2001; Cheng et al, 2003).

Because the amount of MSCs is too low (0.001% -0.01%) in adult bone marrow (Pittenger et al, 1999), the best way to obtain sufficient MSCs is in expanding *ex vivo* culture. In this study, the biological properties of adult MSCs isolated from non-human primates were characterized. The results indicated Rhesus MSC cells displayed a stable phenotype and remained as a monolayer *in vitro*. The growth kinetics of these cell populations revealed rapid adherence and clonal efficiency consist through to passage 4, but it decreased in passage 5 and higher. However the cell proliferation rate decreased in passages 4 and 5 compared with the earlier passages. In passage 5, the cell proliferation obviously slowed down, and the cell morphology was triangular, polygon or flat, which was similar to Cheng et al (2003).

MSCs have been reported to require specific culture and propagation conditions and other supplements for long-term culture. Multiple factors, such as medium and serum selection, seeding ratio and micro-environment of the culture (pH and temperature), can affect the cell yield and passage. In our experience, MSCs can be isolated by their adherence properties to plastic in cell culture. We also observed no special requirements for the culture expansion of Rhesus MSC cells. This may explain the relatively slow doubling capacity of the MSCs observed.

To promote proliferation ability and inhibit differentiation, we gently centrifuged the isolated mesenchymal cells to form a pelleted micromass, cultured the cells with 10% serum and supplemented the fibroblast growth factor (bFGF). Ten percent serum supports cell growth without providing redundant inducing factor that can lead to over-differentiation. bFGF has the ability to induce cell growth while blocking differentiation, and can compensate for the differences between sera lots which influence the cell growth ability. Moreover, considering the growth of MSCs depends on population patterns, the low splitting ratio could be a critical factor for passage. Our data demonstrated that a 1∶2 seeding ratio can assist cells to adapt in *ex vivo* culture.

In summary, the rhMSCs described here have the ability to proliferate extensively, and they maintain the division ability up to passage 5 *in vitro*, suggesting that bone marrow will prove to be an important source of pluripotent stem cells. Whereas this study only reports on the morphology of rhMCSs, more studies of cell surface markers and flow analysis should be conducted. Moreover, detailed investigations on maintaining stem cell activity and extending passages should also be addressed.

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